STUDIES ON SHELL FORMATION. X. A STUDY OF THE PROTEINS OF THE EXTRAPALLIAL FLUID IN SOME MOLLUSCAN SPECIES[1, 2]

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The crystals and organic matrix of shell are formed within a thin layer termed the extrapallial fluid (de Waele, 1930) enclosed between the mantle and the inner shell surface. Accordingly, this fluid is considered to contain precursors of the organic matrix, calcium carbonate in the state just prior to its deposition, carbon dioxide, and other substances. At the same time, it is probable that physico-chemical characteristics and composition of the fluid will determine the nature of the organic matrix and type of calcium carbonate crystals deposited, that is, whether aragonite, calcite, or vaterite.

Recently evidence has accumulated on the relation of the organic matrix of shell to the shell layers, to taxonomic groups, and to the crystallization of calcium carbonate. For instance, the proteins contained in the prismatic and nacreous layers of a shell (Roche *et al.*, 1951; Hare, 1963) and also those in the prismatic and nacreous portions of cultured pearls (Yasuda *et al.*, 1957; Tanaka *et al.*, 1960) differ in the amino acid composition of the parts. Grégoire *et al.* (1955) and Grégoire (1957) have demonstrated a difference of the lace-like pattern of the organic matrix of the nacreous layer in the various groups of molluscs. Watabe and Wilbur (1960) have reported experiments suggesting that in the shell formation of molluscs the nature of the organic matrix has an important role in determining the crystal forms. Recently, Kitano and Hood (unpublished data) reported the effect of organic material upon the crystal forms of calcium carbonate *in vitro*.

While the importance of the extrapallial fluid in shell formation has been emphasized by de Waele (1930), Robertson (1941), Stolkowski (1951), and others, most of the previous investigations in this field have been concentrated upon the characteristics of the mantle and shell itself, omitting consideration of this fluid. Accordingly, our knowledge concerning the protein components of the extrapallial fluid is almost completely lacking. The present experiments were undertaken, as an initial study in this field, to detect the distribution of these proteins in some molluscan species by means of paper and cellulose acetate electrophoresis.

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Materials and Methods

The following fourteen species were used: (1) *Aequipecten irradians concentricus*, (2) *Chlamys nipponensis*, (3) *Crassostrea virginica*, (4) *C. gigas*, (5) *Ostrea edulis*, (6) *O. lurida*, (7) *Modiolus demissus*, (8) *Busycon carica*, (9) *Mercenaria mercenaria*, (10) *Elliptio complanatus*, (11) *Cristaria woodiana*, (12) *Corbicula leana*, (13) *Viviparus intertextus*, and (14) *Cipangopaludina malleata*. Of these, the first nine species are marine and the latter five fresh-water forms. It has been known that in the normal state, calcium carbonate crystals of the shell in the species of (1)–(6) are wholly composed of calcite, those of (7) of calcite in the prismatic layer and of aragonite in the nacreous layer, and those of (8)–(14) wholly aragonite (Bøggild, 1930; Bronn, 1935; Stolkowski, 1951; Lowenstam, 1954; Odum, 1957; Turekian and Armstrong, 1960; Wada, 1961).

Fluids of marine forms were usually taken from animals freshly collected at the Duke Marine Laboratory, Beaufort, North Carolina. In other cases, both marine and fresh-water animals were maintained in the Department of Zoology, Duke University. In the Japanese marine forms, fluids were collected from animals which had been cultured in hanging cages at a depth of about 1.5 meters in Hakodate Harbor, following a manner similar to the pearl-oyster culture; fresh-water forms were reared in tanks of a green-house of the Faculty of Fisheries, Hokkaido University.

For collection of the extrapallial fluid, the following method was simplest and most satisfactory. After opening the valves by means of a shell speculum used in pearl culture, a tiny hole was made by separating some pallial muscle from the inner surface of the shell. A long injection needle on a syringe was inserted through this hole into the extrapallial space, always being careful that the tip of the needle was in contact with the inner surface of the shell. The extrapallial fluid was withdrawn by gentle suction. In the case of *Busycon carica, Viviparus intertextus* and *Cipangopaludina malleata*, the fluid was collected in a similar manner by inserting a needle into the extrapallial space and withdrawing fluid after the foot had been extended. Usually, fluid collected from 2–10 specimens was pooled and used for each test. Blood was carefully collected by heart puncture and gentle suction applied at the time of each beat.

The extrapallial fluid was centrifuged and the supernatant was concentrated to about one-tenth its original volume by dialysis against 20% gum arabic for about 20–30 hours at 4° C. During dialysis, a whitish precipitate formed in the concentrated fluid. No distinct difference of the electrophoretic pattern was found between the runs on the supernatant only and the mixture of both fluid and precipitate. Blood was concentrated to about one-third its original amount by dialysis against 10% gum arabic at 4° C. for about 16–20 hours.

An E–C Apparatus Co. paper electrophoresis system was used. Most of the procedures were performed according to the recommendations made by Raymond (1955) for this apparatus. In most cases, six strips were run simultaneously, including a control of human serum. On the basis of preliminary experiments, the following conditions were chosen: barbital buffer solution (Holt *et al.*, 1952), pH 8.5 and ionic strength 0.045; delivered constant current intensity 0.8 mA/cm. width of the strip, and voltage-gradient during each run about 10–11 V/cm. length of the

strip; cooling temperature of the apparatus 10° C.; duration of run 4.5 hours; Whatman 3MM filter paper strip of 3.1 × 46.5 cm.; a sample of 0.05–0.1 ml. was applied to the strip at its origin as a straight streak 1.5 cm. wide with a microsyringe. The amount of human serum applied was 0.01 ml. When a relatively high current intensity was delivered, a better electrophoretic pattern was obtained, possibly owing to high viscosity of the concentrated extrapallial fluid applied, though such a condition was not desirable for human serum used as a control. After completion of the electrophoresis, the strips were dried at about 100° C., rinsed in methanol for 10 minutes, and stained for 30 minutes in a 0.1% solution of bromphenol blue in methanol; after washing successively in three changes of 2% acetic acid, the strips were dried in air; the color was intensified by exposure to ammonia vapor and the strips were then scanned with a Spinco Model RA Analytrol.

The paper electrophoresis on the fluid of *Crassostrea gigas, Ostrea edulis, O. lurida, Chlamys nipponensis, Cristaria woodiana, Corbicula leana,* and *Cipangopaludina malleata* was conducted in Japan under the following conditions: barbital buffer solution, pH 8.5 and ionic strength 0.045; delivered constant current intensity 0.5 mA/cm. width of the strip, and voltage-gradient during each run about 10–12 V/cm. length of the strip; cooling temperature of the apparatus 15° C.; duration of run 3–3.5 hours; Toyo No. 51 filter paper 12.5 × 26 cm. (four simultaneously run); amount of sample 0.05–0.1 ml. A modified type of Grassman-Hannig's electrophoresis apparatus was used. Scanning of the strips was made with a Natsume densitometer after clearing them in melted paraffin.[4]

Electrophoresis on cellulose acetate was made under the following conditions in which most of the procedures were performed according to Smith (1960): Laurell's barbital buffer solution, pH 8.6, 0.07 *M* solution for impregnation of the strips and 0.06 *M* for electrophoretic run; strip size 2.5 × 18 cm., connected with two filter paper strips of 15 cm. length and 2.5 cm. width which were long enough to reach down into the buffer solution in tanks on both sides of the apparatus. Certain practical difficulties were encountered in the use of cellulose acetate strips. For instance, the sample was hardly absorbed on the strip when applied, owing to its high viscosity; it was not rare that it took one or two minutes to absorb. So, even if the sample was applied to the cellulose acetate strip so as to be linear at its origin, by use of the microsyringe, usually it spread around very slowly, forming a round spot. Accordingly, it was difficult to obtain a satisfactory electrophoretic pattern, especially with samples from species having complex protein fractions. In spite of such disadvantages, separation of the protein fractions was found to be better than that obtained from the paper electrophoresis.

Results

The electrophoretic patterns of the extrapallial fluid for each species are presented in Figures 1 and 2. Generally, separation of the protein fractions by paper electrophoresis was not clear as in the case of the human serum, possibly owing to abundance of mucoidal substances. However, as is seen in Figure 1 B, the

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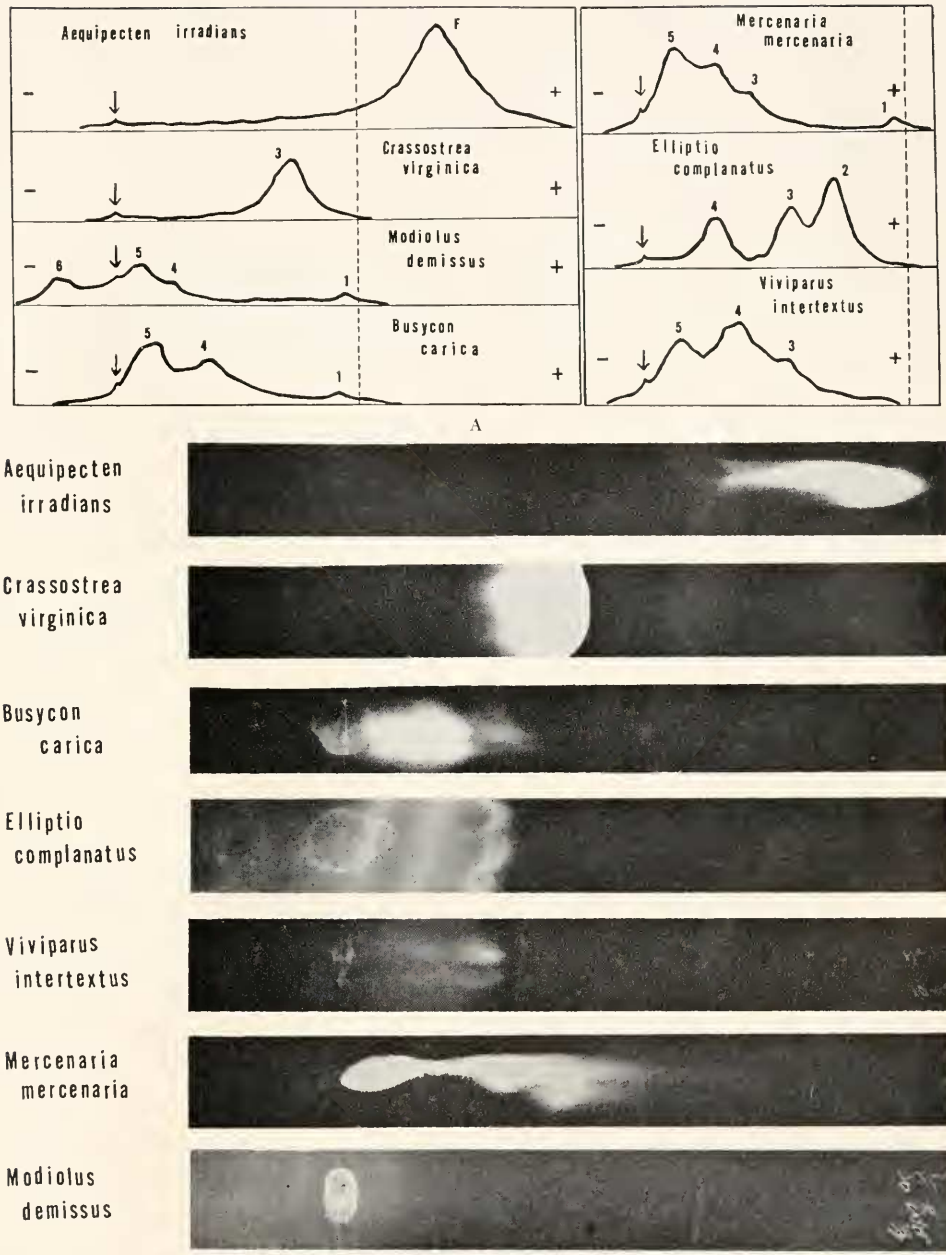

FIGURE 1. Electrophoretic patterns of the proteins of the extrapallial fluids in seven molluscan species. A: scannings obtained from paper electrophoresis; arrows indicate the origin of the strip; dotted line the relative migration distance of albumin of human serum. B: cellulose acetate electrophoretic diagrams of the proteins of the extrapallial fluids; all strips were stained with Ponceau S, but not cleared, and printed directly on photographic paper.

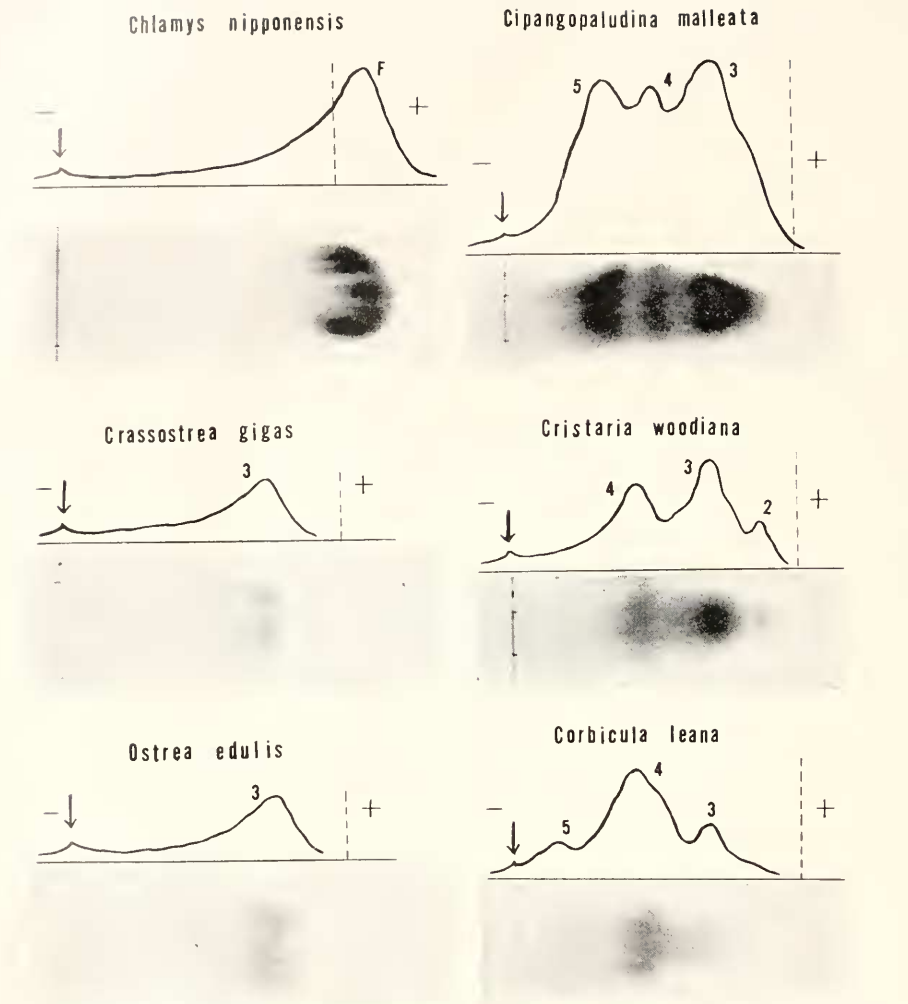

FIGURE 2. Paper electrophoretic patterns of the fluid proteins in six molluscan species. Dotted line indicates the relative migration distance of albumin of human serum.

cellulose acetate electrophoresis showed a relatively clear separation, although some fractions were found to show trailing and further, strictly speaking, migration distances of some fractions were not comparable with those of the paper electrophoresis. Nevertheless, from both the scannings and the diagrams shown in Figures 1 and 2, one is able to identify the individual protein fractions in each species. The relative migration distances of the protein fractions for each species are indicated in Table I. The variation in the migration rate of each fraction was considerable. However, except for the oysters, the species specificity of the electrophoretic patterns was always unmistakable. The migration rates of fractions of human serum are indicated in Table I for comparison.

A single protein fraction was found in the extrapallial fluid of *Aequipecten irradians concentricus, Chlamys nipponensis, Crassostrea virginica, C. gigas, Ostrea edulis*, and *O. lurida* (Figs. 1 and 2; diagram of the last species not presented). The occurrence of such a single fraction in these species was remarkably constant in every run, although it usually occupied a considerable area rather than appearing as a clear band. The fraction in the scallops, *Aequipecten irradians concentricus* and *Chlamys nipponensis*, corresponded to the so-called F-fraction, though the migration velocity of the former species was much faster than that of the latter (Table I). In all of the oysters, *Crassostrea virginica, C. gigas, Ostrea edulis* and *O. lurida*, the fraction was found at a position similar to alpha-2-globulin of human serum. Accordingly, it is probable that the two fractions of the two groups, scallops and oysters, differ chemically. This is further suggested by a characteristic difference in pattern shape on cellulose acetate (Fig. 1 B); that is, in *Aequipecten irradians concentricus*, the fraction was always found to show trail-

TABLE I

Relative migration distance of each protein fraction in extrapallial fluid and blood of fourteen molluscan species, calculated from the scannings of paper electrophoresis

Species	Fluids	No. of samples used	Protein fractions						
			F	1	2	3	4	5	6
Aequipecten irradians concentricus	Ex	14	144 ±9.4						
	Bl	9	123 ±5.4						
Chlamys nipponensis	Ex	8	107 ±7.1						
	Bl	10	111 ±5.1						
Crassostrea virginica	Ex	19				71 ±6.6			
	Bl	12				69 ±5.5			
Crassostrea gigas	Ex	9				68 ±3.9			
	Bl	2				66			
Ostrea edulis	Ex	7				67 ±3.1			
	Bl	2				70			
Ostrea lurida	Ex	4				70 ±2.2			
Busycon carica	Ex	17		94 ±4.8			44 ±6.3	16 ±3.0	
Elliptio complanatus	Ex	17			85 ±6.1	60 ±6.0	25 ±4.1		
	Bl	9			85 ±7.0	57 ±8.8	19 ±6.1		
Cristaria woodiana	Ex	19			86 ±5.5	66 ±5.8	36 ±3.2		
	Bl	5			81 ±3.0	64 ±1.8	33 ±6.5		
Corbicula leana	Ex	6				65 ±2.0	44 ±4.4	16 ±2.6	
Viviparus intertextus	Ex	8				64 ±4.6	48 ±5.2	17 ±1.5	
Cipangopaludina malleata	Ex	6				70 ±2.9	50 ±2.9	30 ±5.8	
Mercenaria mercenaria	Ex	20		98 ±4.5		41 ±7.1	19 ±4.2	11 ±3.5	
	Bl	9			85 ±5.2	39 ±4.6	23 ±4.5	9 ±2.2	
Modiolus demissus	Ex	15		95 ±6.5			24 ±7.3	10 ±3.5	−12 ±5.4
Human serum (control)		42		Alb 100	α_1 84 ±2.7	α_2 62 ±3.6	β 38 ±3.4	γ 11 ±2.0	

Fluids: Ex, Extrapallial fluid; Bl, Blood.
For each fraction, the value (±S.D.) for the migration distance is given relative to that of human serum albumin as 100%.

ing on cellulose acetate electrophoresis which was made under the same conditions as for *Crassostrea virginica*.

In each of the seven species, *Busycon carica*, *Elliptio complanatus*, *Viviparus intertextus*, *Cristaria woodiana*, *Corbicula leana*, and *Cipangopaludina malleata*, three fractions were found (Figs. 1 and 2). In all diagrams of *Busycon carica*, fraction 1 was found to be so faint as to be scarcely visible on paper after exposure to ammonia vapor, though it was clearly detectable on cellulose acetate strips. In almost all diagrams of *Elliptio complanatus* obtained by the cellulose acetate electrophoresis, the fastest fraction appeared to resolve itself into two fractions (Fig. 1 B). Although the occurrence of such an additional fraction was not distinctly recognizable in paper electrophoresis, four protein fractions may be present. In *Viviparus intertextus*, fraction 3 was not clear-cut on paper but was a distinct narrow band on cellulose acetate (Fig. 1 B). In all the Japanese three species, *Cristaria woodiana*, *Corbicula leana*, and *Cipangopaludina malleata*, three protein fractions were clearly identified, each species showing the respective specific pattern (Fig. 2). In the last species, the fastest fraction appears to resolve itself into two fractions when considered from its scanning pattern.

Among the 14 species studied, the electrophoresis of the extrapallial fluid in *Modiolus demissus* was most difficult. No satisfactory pattern was obtainable, possibly owing to its low concentration of total protein. And when the sample was concentrated more than 15 times, it was not easily absorbed on the filter paper, and especially on the cellulose acetate strip, because of its high viscosity. However, the scanning pattern of this species shown suggests four protein fractions, including one (fraction 6) which migrated towards the anode (Fig. 1 A). In most cases of paper electrophoresis in this species, fraction 1 was found to be so faint as to be scarcely visible after exposure to ammonia vapor, but it appeared as a relatively clear and slender band on cellulose acetate strips (Fig. 1 B). In *Mercenaria mercenaria* also, the four protein fractions are suggested but cannot be clearly resolved. Fraction 1, a minor component, scarcely detectable on paper, was always found to show trailing as a characteristic long tail-like appearance on cellulose acetate (Fig. 1 B).

The number of protein fractions in the blood was the same as in the extrapallial fluid in the eight species examined. However, as indicated in Table I, in *Aequipecten irradians concentricus* and *Mercenaria mercenaria* there were differences in migration velocity between blood and extrapallial fluid. The pericardial fluid of *Chlamys nipponensis*, *Crassostrea gigas*, and *Ostrea edulis* showed a single fraction is in both the extrapallial fluid and the blood in each species (diagrams not presented).

Discussion

It is clear from the present experiments that the extrapallial fluid in the species with a calcite shell (*Aequipecten*, *Chlamys*, *Crassostrea*, and *Ostrea*) contains a single protein fraction, while species with an aragonite shell (*Busycon*, *Mercenaria*, *Elliptio*, *Viviparus*, *Cristaria*, *Corbicula*, and *Cipangopaludina*) or a shell of both aragonite and calcite (*Modiolus*) have three or more protein fractions. The species studied are few in number; however, these results suggest the possibility that extrapallial fluid which possesses a complex system of protein components may

favor aragonite formation, while that having a single protein component may crystallize as calcite during shell deposition.

Yago *et al.* (1959) have reported that both the blood and the pericardial fluid in *Crassostrea gigas* revealed a single protein fraction, in agreement with the present experiments. Woods *et al.* (1958) have reported that the serum of *Crassostrea virginica* showed two slow components, using starch gel electrophoresis. However, the writer's preliminary experiments made on the same species suggest that some slow components are often found to occur in the following cases: in extrapallial fluid collected from a specimen in which the mantle was intentionally injured by the tip of the needle, and in blood collected from a relatively small heart by strong suction with the syringe. Further, paper electrophoresis of minced tissue fluid in this species also showed the occurrence of such slow components.

The technique of paper partition chromatography has been applied to the taxonomic study of molluscs. Wright (1959) and Collyer (1961) have reported the biochemical specificity in mucus and tissue fluid. The ninhydrin-positive substances studied by these investigators, and protein components in the extrapallial fluid, showed a remarkable degree of specificity except in the species of the oysters studied by electrophoresis. The specific fenestration of the organic matrix in the shell (Grégoire *et al.*, 1955) might be a pattern formed in relation to such a biochemical specificity of the extrapallial fluid in molluscs. Accordingly, both *in vitro* and *in vivo* studies which may show a correlation between the organic matrix, as synthesized from either a simple or a complex system of protein components and the crystal type of calcium carbonate, may offer some evidence on the mechanism of shell formation. It is interesting to note here that such a preliminary *in vitro* experiment has been made by Kitano and Hood (unpublished data).

Summary

1. Distribution of the protein fractions of the extrapallial fluid of fourteen species of molluscs was determined by means of paper and cellulose acetate electrophoresis.

2. The extrapallial fluid of species in which the calcium carbonate of the shell occurred as calcite contained a single protein fraction. In those species in which the shell calcium carbonate occurred as aragonite or as both aragonite and calcite, three or more protein fractions were present in the extrapallial fluid.

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